Received: 3 December 2020 | Revised: 24 May 2021 | Accepted: 13 June 2021

DOI: 10.1002/tox.23310

RESEARCH ARTICLE

ENVIRONMENTAL TOXICOLOGY WILEY

Toxicity of malathion during Senegalese sole, *Solea senegalensis* larval development and metamorphosis: Histopathological disorders and effects on type B esterases and CYP1A enzymatic systems

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Funding information
Ministry of Science and Innovation (MICIIN) of the Spanish government, Grant/Award Number: AGL2010-15951

Abstract

The toxicity of malathion to *Solea senegalensis* was studied in a static renewal bioassay during its first month of larval life (between 4 and 30 dph). Through the use of different biomarkers and biochemical, cellular and molecular approaches (inhibition of cholinesterases [ChEs], changes in cytochrome P450-1A [CYP1A] and the study of histopathological alterations), the effects of three concentrations of malathion (1.56, 3.12, and 6.25 μg/L) have been analyzed. In subacute exposure, malathion inhibited cholinesterase activities (AChE, BChE, CbE) in a dose- and time-dependent manner, ranging the inhibition percentage from 20% to 90%. However, the expression levels of CYP1A and AChE transcripts or proteins were not modified. Additionally, exposure to malathion provoked histopathological alterations in several organ systems of Senegalese sole in a time- and dose dependent way, namely disruption of parenchymal architecture in the liver, epithelial desquamation, pyknotic nuclei and steatosis in the intestine, disorganization of supporting cartilage, and sings of hyperplasia and hypertrophy in the gills and degeneration of the epithelial cells from the renal tubules. Malathion exposure also provoked strong disorganization of cardiac fibers from the heart. The findings provide evidence that exposure to sublethal concentrations of malathion that provoked serious injury to the fish *S. senegalensis*, were below the expected environmental concentrations reported in many other ecosystems and different fish species,revealing a higher sensitivity for Solea senegalensis to malathion exposure, thus reinforcing its use as sentinel species for environmental pollution in coastal and estuarine environments.

KEYWORDS
CYP1A, Flatfish, histopathology, organophosphates, type B esterases

1 | INTRODUCTION

The relationship between environmental stress and diseases is increasingly well established over time.[1] Among the natural or human-induced environmental factors that can have serious effects on fish health, water pollution has been considered the most important.[2] In this context, the early life stages of fish in contaminated areas are more vulnerable to predation.[3] Some agrochemical xenobiotics (e.g., insecticides) can induce harmful alterations, such as imbalances in neurotransmitters and alterations in the development of the thyroid and dependent hormonal dysfunctions, which are responsible for behavioral alterations and the induction of pathologies and diseases.[3–5] In different species in contaminated ecosystems, as well as in numerous experimental toxicity studies in fish, including the Senegalese sole, different neuroendocrine alterations, skeletal deformities, and pigment abnormalities, as well as serious histopathological alterations in most critical organs and tissues have been recorded. In addition, low growth and survival patterns and even high mortality rates have been registered in fish growing under high-stress conditions.[6–9]

Organophosphates (OPs) form a large group of chemicals used over the past 60 years to protect crops, livestock, human health and as warfare agents.[10] Despite its prohibition by the European Commission since June 2007,[11] malathion [1,2-di(ethoxycarbonyl) ethyl *O*,*O*-dimethyl phosphorodithioate], which is a broad spectrum organophosphate insecticide is still used worldwide, causing serious problems in coastal areas, from agricultural, urban, and industrial sources. Malathion is a phosphorodithioate compound that is particularly toxic to most insects, and has moderate to high toxicity in vertebrates, where concentrations as low as four parts per billion (ppb) have been shown to adversely affect the fish.[12] Among many other lipophilic xenobiotics, OP insecticides can reach natural waters by transferring chemicals, from the ground or by direct spraying to target and non-target species, such as fish, crustaceans, and mollusks.[13–17]

The acute toxicity of OPs in different species depends on the relative levels of their metabolic activating and detoxifying enzymes, such as monooxygenases (i.e., cytochrome P450 [CYPs]), type B-esterases, glutathione S-transferases, and so forth.[5,13,14] The activity and expression of different CYPs involved in malathion metabolism and its toxicity show inter and intra-specific differences. In mammals, different families of CYP isoforms (i.e., CYP1A, CYP2B, CYP3A, etc.) play a critical role in the toxicity of OPs, like malathion, as various CYPs catalyze the formation of their oxon-metabolites.[18–20] The main bioactive metabolite of malation, malaoxon, provides greater inhibitory potency in different enzymatic pathways, such as acetylcholinesterase (AChE), carboxylesterase (CbEs), and CYPs.[18,21–23] In contrast, in fish species there are interspecific variations in activity and expression of CYP's involved in malathion metabolism.[24–27]

Cholinesterases (ChEs) inhibition is the most critical toxic effect of OPs exposure. The inhibition of AChE results in the accumulation of neurotransmitter acetylcholine (ACh) in the synapse, interrupting the normal transmission of neuronal signals.[3,5,13] The CbE catalyzes the release of endogenous or exogenous short chain fatty acids, which intervene in the metabolism of xenobiotics, favoring their detoxification.[5,28] The physiological role of butyrylcholinesterase (BChE) is less well known. This enzyme has been shown to promote the release of fatty acids from triacylglycerol and phospholipids, and it can also act as a detoxifying enzyme.[29,30] Both enzyme systems, CYPs and CbEs, can compete for malathion as a substrate, and are responsible for metabolizing OPs into the products of their initial metabolic degradation.[5,28] It has also been suggested for CbE, a protective role in resistance and/or defense mechanisms against the inhibition of AChE induced by OP pesticides.[24–26,28,31]

On the other hand, histopathological investigations have been increasingly recognized as an important tool for the assessment of the impact of environmental pollutants on aquatic animals. These can be used as biomonitoring tools or health indicators in toxicity studies, as they provide early warning signs of disease and the health of the aquatic environment.[32] In toxicological studies, histopathology is primarily aimed at determining the effect of contaminants on structural components of the living system and the ways in which cells and tissues respond to injury. When a contaminating compound acts directly on cells, it causes chemical cytotocicity by altering their environment. As a consequence, cells respond histopathologically by degeneration, proliferation, and repair. All these cell and tissue injuries and damages that appear after contaminants exposure can significantly alter the function of tissues and organs, resulting in reduced survival, growth, and fitness of affected fish. Several studies have emphasized the toxic effects of exposure to malathion in different fish species such as, behavioral alteration and respiratory dysfunction in *Labeo rohita*,[33] histochemical and histological alterations in ovary, brain, and liver of *Ophiocephalus punctatus*,[34] histopathological alterations in kidney, ovary, and liver of *Heteropneustes fossilis*,[35] hematological changes and morphological alterations in the gills of *Cyprinus carpio*,[36] among others.

Unlike the large amount of information available on the toxicity of pesticides on freshwater organisms, relatively little data exists on the effects of these insecticides on marine and estuarine organisms. There have been some studies that analyze the toxicity of pesticides in different species of commercial and farmed marine teleosts,[37,38] enclosing commercial flatfish species. However, investigations related to the toxicity of pesticides in bentic species like *Solea senegalensis*, are still scarce.[8,16,39–42]

Several field studies have reported the presence of malathion in water samples at concentrations ranging between 18.12,[43] 29.84,[44] and 105.2 μg/L.[45] Based on these considerations, the objective of the present study was to analyze the chronic effects of environmentally relevant concentrations of malathion, which were previously identified as sublethal doses for this OP insecticide in *S. senegalensis*,[42] through the use of different approaches and biochemical, cellular and molecular biomarkers. The integrative effects have been analyzed against an aquatic exposure to three nominal concentrations of malathion (1.56, 3.12, and 6.25 μg/L) during the first month of larval life (from 4 to 30 dph). Changes in enzyme patters (cholinesterases, Cyps), at the molecular, biochemical, and cellular levels, as well as histopathological alterations and cell proliferation (proliferating cell nuclear antigen [PCNA]) versus apoptosis (TUNEL assay) in different organs were carried out in experimental groups in an attempt to elucidate the impact of sublethal and environmentally relevant malathion exposure concentrations.

2 | MATERIAL AND METHODS

2.1 | Larval rearing and experimental assays

Newly hatched larvae of *S. senegalensis* (provided by the IFAPA-El Toruño; Regional Government of Andalusia, Spain) were reared at ICMAN-CSIC (Puerto Real, Cádiz, Spain). The larvae (initial larval density 50–60 larvae/L) were distributed in fifteen 16 L cylindroconical tanks, and maintained from hatching to 30 days post-hatching (dph) under standard rearing conditions. Our facilities, in accordance with the European Convention for the Protection of Animals used for Experimental and Scientific Purposes were approved for experimentation by the Ministry of Agriculture and Fisheries (REGA-ES110280000311) in accordance with the European Directive and following the Spanish normative (RD 1201/2005). The experimental procedure (Proyect AGL2010-15951) was approved by the Spanish National Research Council (CSIC) Etical Committee, and the Etical Committee for Animal Welfare of ICMAN-CSIC.

The water temperature was maintained at 20 ± 0.4°C, the salinity 32–38 g/L, and the dissolved oxygen at 85%–100%. The photoperiod was maintained at 12 h light:12 h dark (light intensity of 600–800 lx) and gentle aeration was provided. The larvae were fed with rotifers from 3 to 9 days post-hatching (dph) at a density of 15 organisms per ml and artemia nauplii from 6 dph at an initial density of one organism per ml. The density of artemia increased gradually, becoming the only prey added from 5 dph onwards, according to previous studies.[46,47]

Malathion (C10H19O6PS2; >98% purity) was obtained from Sigma-Aldrich Chemical Co (St Louis). The malathion challenge tests consisted of a control, a solvent control and three malathion concentrations (1.56, 3.12, and 6.25 μg/L, nominal concentrations). These concentrations were previously tested as sublethals (below the LC50 72 h, for *S. senegalensis*) according to Ortiz-Delgado et al.[8]

To perform the exposure tests, the cylindroconical tanks were randomly assigned in triplicate as control groups and as malathion-exposed groups (control + control/carrier + 3 concentrations of malathion = 5 conditions × triplicate = 15 experimental tanks). Larvae at 4 dph and during the first moth of life (day 30) were exposed to malathion in semistatic-renewal toxicity tests (50% renewal every 24 h). Both the controls and the exposure treatments received the same carrier solvent (acetone 0.01%, maximum acceptable limit for the solvent with no observable effect, according to OECD guidelines[48,49]). All the experiments were carried out with Senegalese sole larvae from 4 dph onwards, in order to avoid the high natural mortality, which is very common, during the endogenous feeding phase of this flatfish species. At the end of the experiment, no differences in mortality were observed between controls and the groups exposed to malathion (survival rate of 75%–85% at 20–30 dph).

2.2 | Histological procedures

For histological purposes, samples of Senegalese sole larvae ($n = 3$ fish per each experimental tank) were collected at 13, 20, and 30 dph (3 larvae × 15 tanks × 3 sampling points = 135 larvae), anesthetized with 500 ppm phenoxyethanol and processed following a RNAse-free procedure, according to previous studies.[47,50] Once collected and anesthetized, samples were fixed with 4% freshly-prepared paraformaldehyde in diethyl pyrocarbonate-(DEPC)-treated phosphate buffered saline (PBS) ×1 overnight at 4°C. Afterwards, samples were stored in methanol at −20°C after washing three times for 1 h with PBS ×1, and then processed following our standardized technical protocols.[47,50] Briefly, samples were embedded in paraffin following a standard protocol to obtain 5–6 μm histological serial sections of whole larvae.

2.3 | Histopathological alterations

The Hematoxylin-Eosin and Hematoxylin-VOF techniques[51] were used on deparaffinized slides to verify the histomorphological characterization of larval development and the presence of histopathological alterations caused by exposure to malathion.

The evaluation of the histopathological results was carried out independently by four observers, who analyzed the same histological samples without knowing their origin. Histological results were visualized under a light microscope (Leitz diaplan) and recorded manually on a table. The quantification of the histological alterations was performed in 60 non-consecutive slide sections for each treatment and controls. For this, 15 slides containing four sections of three individuals (included in the same paraffin block) from each experimental tank were observed, registering the presence of histological alterations in target organs (liver, kidney, intestine, gills, and heart). Results were expressed as the semi-quantitative assessment of the percentage of affected fish.

2.4 | Immunohistochemistry

The immunohistochemical approach was performed in histological sections of at the end of the exposure period (30 days), using the commercial primary antibodies CYP1A (Biosense, monoclonal mouse anti-rainbow trout CYP1A), PCNA (Santa Cruz Biotechnology, monoclonal mouse anti-rat PCNA), and AChE (abcam, monoclonal mouse anti-human AChE) according to Ortiz-Delgado et al.[47] At the same time, to confirm the specificity of the primary antibodies and the immunostaining results, positive and negative controls have also been tested; for instance, replacing primary antibody with preimmune serum or bobine serum albumin and incubating only with secondary antibody (Vector, biotinylated horse anti-mouse IgG) as well as omitting the secondary antibody.

2.5 | TUNEL assay

Cell apoptosis in control and exposed fish was determined by TUNEL method in histological sections at the end of the experimental period (30 dph), by using the in situ cell death detection Kit (Roche Life Sciences), which identifies DNA damage at 3′-OH endings.[52] The slides were treated with proteinase K (10 μg/ml in 10 mM Tris HCl, at pH 7.4) for 30 min at 37°C, washed in PBS, followed by incubation in 0.3% H_2O_2-containing methanol for 30 min at room temperature to block the endogenous peroxidase (POD) activity. After washing in

PBS, the slides were incubated with 5 μl of enzyme solution and 45 μl of labelling solution (BrdU FITC) for 1 h at 37°C in the dark. Two controls were used: a positive control where slides were treated with DNase I (3 U/ml in 50 mM Tris HCl, pH: 7.5) during 10 min, previously to the labelling and a negative control where slides were incubated without the enzyme solution. Slides were visualized in a fluorescent microscope (Leica DM 2000, Germany) with 450–490 nm excitation and 515 nm emission wavelengths. To transform fluorescence-based TUNEL assay into a colorimetric assay suitable for light microscopy, an anti-fluorescein antibody (Fab fragments from sheep) conjugated with horse-radish POD was used. To make the apoptotic cells visible, sections were incubated with 3′-diaminobenzidine as a chromogen.

2.6 | RNA isolation and cDNA synthesis

Pools of larvae of different ages (from 4, 11, 13, 15, 20, and 30 dph) were collected, washed with DEPC water, frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was isolated from 40 mg of pooled larvae using NucleoSpin®RNA II kit (Macherey-Nagel, Düren, Germany) coupling with Lysing Matrix D (Q-BioGene) for 40 s at speed setting 6 in the Fastprep FG120 instrument (Bio101), according to the manufacturer's instructions. In all cases, total RNA was treated twice with DNase I using the RNase-Free DNase kit (Qiagen) for 30 min in order to avoid amplification of contaminated genomic DNA. RNA sample quality was checked using Experion (Bio-Rad) and quantification was performed spectrophotometrically. Total RNA (1 μg) from each sample was reverse-transcribed using the iScriptTM cDNA Synthesis kit (Bio-Rad). Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis.

2.7 | Real-time q-PCR

Real-time analysis was carried out on the iCycler (Bio-Rad). Reactions were accomplished in a 25 μl volume containing 2 μl of a 1/10 dilution of cDNA (≈10 ng), 400 nM each of specific forward and reverse primers and 5 μl of SensiFAST SYBR No-ROX (Bioline). Matching oligonucleotide primers were designed for *Ache* and *Cyp1a* genes using the Oligo v6.89 software (Medprobe) and sequence database GeneBank or SoleaDB (Table 1). Amplification of cDNA fragments encoding target genes was verified in previous assays by direct sequencing of PCR products obtained with the same reaction conditions employed in real-time PCR. The real-time amplification protocol used was as follows: initial 7 min denaturation and enzyme activation at 95°C, 40 cycles of 95°C for 15 s, and 68°C for 30 s. Each assay was done in duplicate. For normalization of cDNA loading, all samples were run in parallel using ubiquitin as housekeeping gene with primers SseUB1 and SseUB2 (Table 1) according to Infante et al.[53] To estimate efficiencies, a standard curve was generated for each primer pair based on known quantities of cDNA (10 fold serial dilutions corresponding to cDNA transcribed from 100 to 0.01 ng of total RNA). All calibration curves exhibited correlation coefficients higher than 0.98, and the corresponding real-time PCR efficiencies were around 2.1–2.2 for both all genes. Relative mRNA expression was determined using the $2^{-(\Delta\Delta Ct)}$ method.[54] Results were expressed as mean ± SEM.

2.8 | In situ hybridization

To study the localization of *Ache* and *Cyp1a* gene expression patterns in different cell- types and tissues of the Senegalese sole, in situ hybridization (ISH) was performed on histological sections of controls and malathion exposed fish, according to Ortiz-Delgado et al.[8,50] To synthesize correspondent probes, different PCR fragments around 93–137 bp were amplified using specific primers (Table 1). PCR products were cloned in TOPO-TA vector and sense and anti-sense probes were synthesized using the DIG RNA labelling kit (SP_6/T_7) (Roche diagnostics, Indianapolis) according to the manufacturer's instructions. Specificity to these probes was confirmed by northern blot analysis. In the ISH protocol, detection of the hybridized probe was carried out using alkaline phosphatase-coupled anti-digoxigenin antibody, and the hybridization signals were detected by NBT/BCIP systems according to the manufacturer's instructions (Roche), adding 5 mM levamisole to neutralize endogenous alkaline phosphatase. The controls included hybridization with sense probes, RNase treatments before hybridization and absence of antisense RNA probe/anti-DIG antibody in the incubation medium.

2.9 | Enzymatic analysis of type B esterases

All enzymatic analysis was performed according to techniques described by Arufe et al.[37,55] Frozen larvae (−80°C) of different ages

TABLE 1 Primers used for semiquantification of *Ache* and *Cyp1a* mRNA levels in *Solea senegalensis*

Gene target	Primers	Sequence (5′-3′)	Base origin	Size (bp)
Sse UB (AB291588.1)	SseUB1	AGCTGGCCCAGAAATATAACTGCGACA	256	93
	SseUB2	ACTTCTTCTTGCGGCAGTTGACAGCAC	348	
Sse ACHE (DB109366)	SseAChE1	CTGTTTGACCACCGAGCATCTAACCTG	5	137
	SseAChE2	CGACTCAGTTTCTCCTCCTCTAGGGTGT	141	
Sse CYP1A (GU946412)	SseCYP1A1	AACAATGATATTCCTGAAGGGCTTCGT	35	116
	SseCYP1A2	TTGCCCATTTCAGTGAGACTCAGGTAA	150	

(13, 20, and 30 dph) were homogenized in ice-cold 0.1 M sodium phosphate buffer (pH 7.4), using an Ultraturrax homogenizer and kept on ice during the homogenization (1 min, 20.500 rpm). The crude extracts from pools of whole-body homogenates were used directly as the enzyme source without centrifugation. Cholinesterase activities (AChE, BChE, CbE) in the homogenates were measured by the method of Ellman et al.,[56] as adapted to microplates previously by Arufe et al.[37] Acetylthiocholine iodide (ASCh) and butyrylthiocholine ioide (BSCh) were used as substrates (0.401 mM final concentration). Briefly, 50 μl of larval homogenate was mixed with 250 μl of a mixture containing 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and substrate (30 ml of buffer phosphate, 1 ml of DTNB at 0.01 M and 0.2 ml of substrate 0.075 M). The change of optical density with time (mOD/min) due to TNB production was recorded at 415 nm for 3 min at room temperature with a microplate reader (BioRad Model 680) to estimate substrate hydrolysis, that was determined in the same way in the absence of enzyme. CbE activity was determined using a similar protocol with the substitution of S-phenylthioacetate (PSA) as substrate. PSA was dissolved in absolute ethanol and the final concentration was 1.068 mM.

The protein content of the enzyme extracts was measured by the method of Bradford[57] adapted to microplates using bovine serum albumin as standard. The measurement was performed with the microplate reader at 595 nm. The enzymatic activity was expressed as nmol hydrolyzed substrate/min/mg protein (specific activity), using a molar extinction coefficient (ε) of 13.600 $M^{-1}cm^{-1}$ for the TNB. All enzymatic reagents were supplied by Sigma-Aldrich Química (Madrid, Spain); Merck (Darmstadt, Germany), and Bio-Rad (Madrid, Spain).

2.10 | Statistical analysis

Enzymatic results were analyzed by One-way analysis of variance, followed by Tukey's multiple comparison test. The Shapiro–Wilk's and Barlett's tests were used to test the normality of the data distribution and the homogeneity of the variances respectively. All enzyme analysis and protein determinations were carried out in triplicate. Temporal differences in the gene expression were tested using the non-parametric Mann–Whitney U test followed by a Dunn-Bonferroti post-hoc employing the InsStat v3.0 software. The differences between treatments and their controls at the same age were detected by a t-Student. Significance was accepted for $p < .05$. For statistical evaluation and graphical representations, control and solvent-carrier control were pooled, as they did not differ significantly with regard to the different recorded measurements.

The assessment for immunohistochemical (CYP, PCNA, and AChE) results, TUNEL assay and ISH was carried out by means of a semi-quantitative[52] visual evaluation performed independently by three observers, who analyzed the same histological samples. Similar results were tested and confirmed analyzing 15 samples per each treatment, and registered using (−), (+) and (++) symbols according to the different levels observed: (−) absent, (+) slightly present, and (++) highly present. A total of 60 slide sections were analyzed for each treatment and control.

3 | RESULTS

3.1 | B-esterases (AChE; BChE; CbE)

In controls, the highest AChE and CbE activities were recorded at the end of the metamorphic phase (at 20 dph; Figure 1(B)) compared to larvae that are beginning this metamorphic process (at 13 dph; Figure 1(A)) and with post-metamorphic juveniles (at 30 dph; Figure 1(C)). The inhibition patterns of AChE, CbE, and BChE, induced by malathion and evaluated at 13, 20, and 30 dph, followed a similar trend (AChE > CbE > BChE). Exposure to malathion caused a statistically significant reduction in the activities of both esterases, AChE and CbE, at the highest concentration (6.25 μg/L) in the larvae at 13, 20, and 30 dph (Figure 1(A)–(C)).

The lowest esterase enzymatic activity corresponded to the BChE or plasma pseudo-cholinesterase activity, both in controls and in malathion exposed Senegalese sole, compared to AChE or CbE activities (Figure 1). Malathion induced enzymatic inhibitions ranged from 20% to 90%. At the highest concentration (6.25 μg/L), malathion caused a statistically significant reduction of AChE (51.5%), BChE (61.85%), and CbE (83.93%) in the larvae during the metamorphosis process (at 13 dph; Figure 1(A)). At the end of the metamorphosis (at 20 dph; Figure 1(B)), exposure to malathion caused significant enzymatic inhibition of AChE (37.7%), BChE (89.5%), and CbE (39.39%) at the highest concentration of 6.25 μg/L. Finally, in postmetamorphic larvae (30 dph; Figure 1(C)), exposure to malathion also caused a significant inhibition of AChE (49.02%), BChE (24.85%), and CbE (65.62%) at the highest concentration of malathion tested. In larvae at 13 and 30 dph, AChE and CbE activities increased even in fish exposed to the lowest dose. On the contrary, in the metamorphosed larvae (at 20 dph), both AChE and CbE activities showed significant decreases, with the lowest concentration of malathion (1.56 μg/L). The maximum inhibition recorded was 64.17% for AChE, 83.93% for CbE and 89.5% for BChE at the highest tested malation concentration (Figure 1(A)–(C)).

3.2 | Cellular localization and expression patterns of Ache and Cyp1a

The transcriptional expression patterns of the Ache and Cyp1a genes (Figure 2(A),(B)), were analyzed in both the controls and malathion-exposed fish. In controls, Ache expression showed a constitutive baseline expression pattern with variable decreasing or increasing trends, throughout the larval development, showing the highest Ache expression levels at the end of the metamorphic stage (at 20 dph). In fish exposed to malation, the expression levels of the Ache transcript displayed upregulations in some methamorphic stages (day 11–3.12 μg/L and day 15–1.56, 3.12, and 6.25 μg/L) and transcriptional down-regulations in post-larval stages (at day 30 in those exposed to 6.25 μg/L) (Figure 2(A)). On the other hand, in the controls, the expression levels of the Cyp1a mRNA transcripts showed variable patterns during larval development, with decreasing levels at 4 dph onwards, and a significant increase in Cyp1a expression levels at the

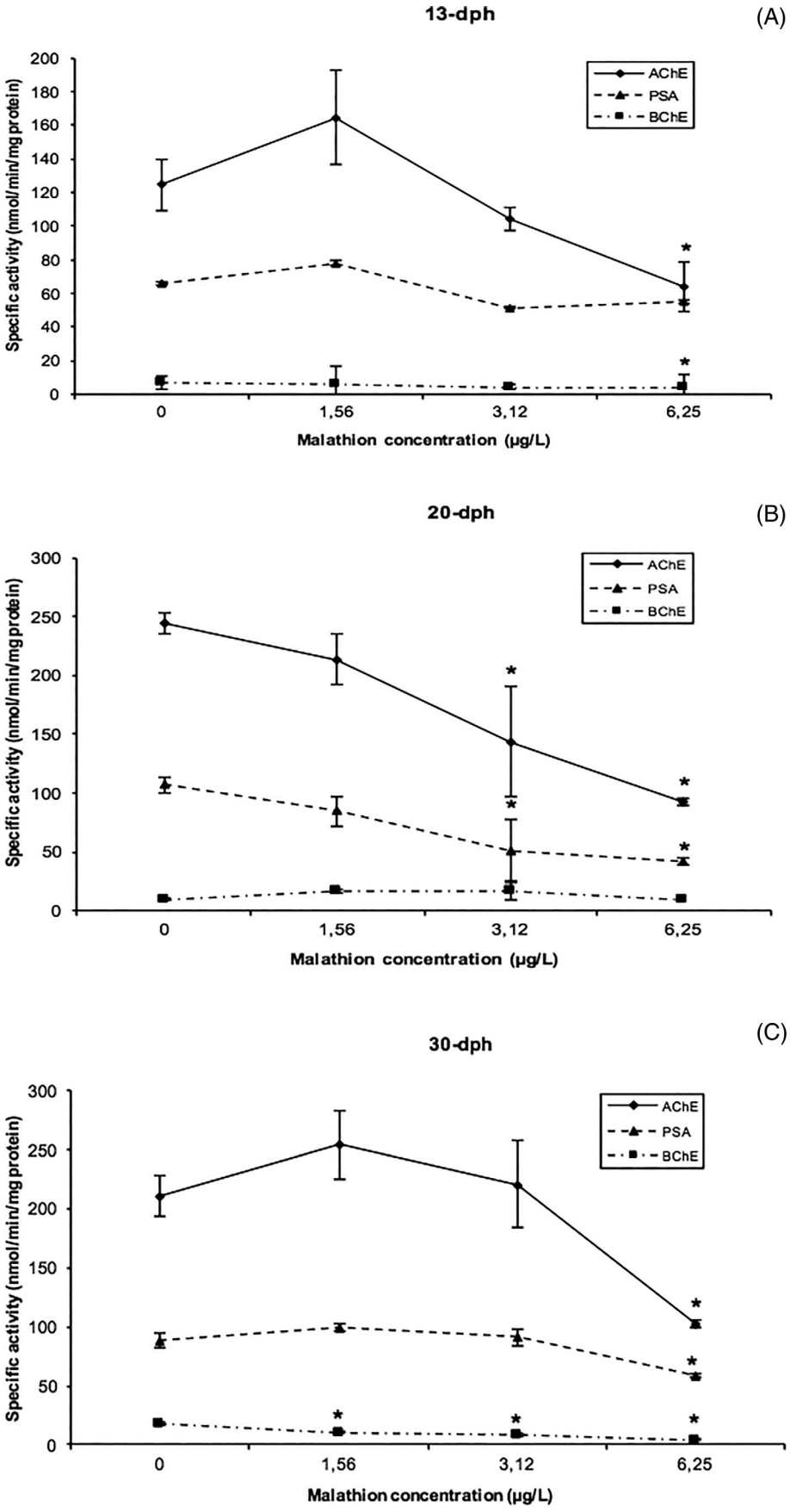

FIGURE 1 Acetylcholinesterase (AChE), carboxylesterase (PSA) and butyrylcholinesterase (BChE) activities in (A) 13, (B) 20, and (C) 30 dph Senegalese sole larvae exposed to different concentrations of malathion. Each point represents the mean response ± SD of three individual experiments for each malathion concentration. Asterisks denote responses significantly different from controls ($p < .05$)

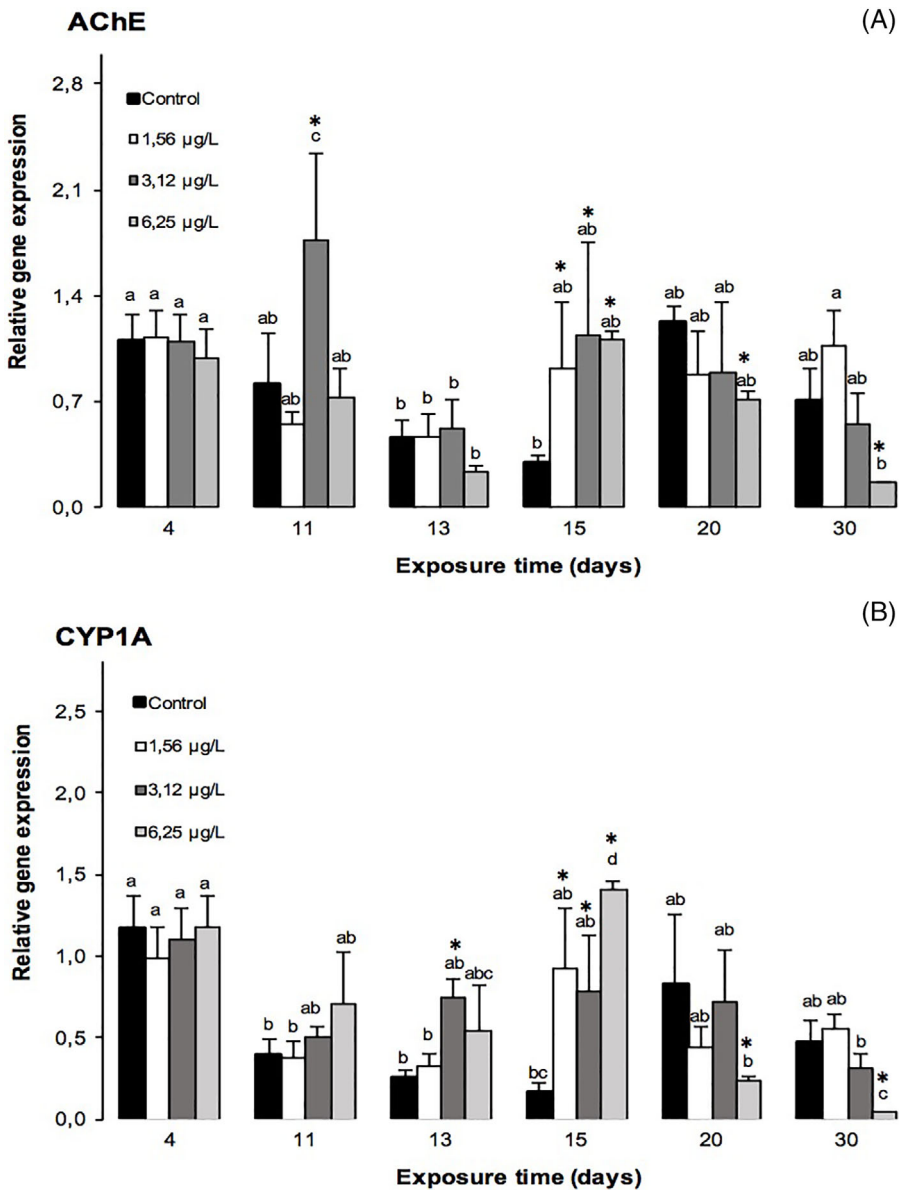

FIGURE 2 Relative Ache (A) and Cyp1a (B) expression levels in Senegalese sole during larval development, in response to the different malathion treatments (mean ± SEM, $n = 3$). Different letters indicate statistically significate differences between treatments at a same age (control 4 dph) ($p < .05$). Values with an asterisk are significantly different (*t*-Student, $p < .05$) from the corresponding value for the controls at the same age

end of the metamorphosis phase (20–30 dph). In the Senegalese sole exposed to malation, transcriptional up-regulations were recorded in Cyp1a expression levels, during some metamorphic stages (at 13 and 15 dph), and transcriptional decreases of Cyp1a, during post-metamorphosis (at 20 and 30 dph in those exposed to 6.25 µg/L) (Figure 2(B)).

The cellular localization of Ache expression by ISH in Senegalese sole control specimens, was remarkable in different brain areas (Table 2). However, no significant detectable changes in Ache expression patterns were detected in larvae exposed to malathion (Table 2). These results were corroborated by immunohistochemistry in which no changes in the tissue distribution of the AChE protein were detected in fish exposed to malathion, when compared with controls (Table 2). Similarly, to what was detected for Ache, Cyp1a cell expression patterns were also not altered by exposure to malathion. Regarding the immunohistochemical distribution of CYP1A in the different organs and tissues (liver, brain, heart, gills, intestine, kidney, among others), no appreciable changes were observed between controls and exposed fish (Table 2).

3.3 | Histopathological alterations in target organs

In Senegalese sole larvae, during the first month of life, exposure to malathion induced histopathological alterations in the liver, gills, digestive system, kidney and heart, as well as changes in the patterns of cell proliferation versus apoptosis. In general, damage to the organ system and cell-tissues progressively increased with malathion concentration and exposure time (Table 3).

The histopathological changes induced in the liver were represented mainly by the alteration of the parenchymal architecture with hepatocellular shrinkage and severe vascular dilation giving the appearance of a spongy-like structure with empty spaces that replaced the liver parenchyma (Figure 3(A),(B)). Hepatocytes lost their

TABLE 2 Cellular localization of Ache and Cyp1a mRNAs and of AChE and CYP1A proteins in control and malathion treated fish at 30 dph

Ache mRNA/AChE protein		**Control**	**1.56**	**3.12**	**6.25**
Brain	Cerebellum	+/+	+/+	+/+	+/+
	Opic tectum	+/+	+/+	+/+	+/+
	Medulla oblongata	+/++	+/++	+/++	+/++
Trunk muscle		+/++	+/++	+/++	+/++
Cyp1a mRNA/CYP1A protein		**Control**	**1.56**	**3.12**	**6.25**
Brain	Olfactory bulb	+/++	+/++	+/++	+/++
	Cerebellum	+/+	+/+	+/+	+/+
	Optic tectum	+/−	+/−	+/−	+/−
	Medulla oblongata	+/−	+/−	+/−	+/−
Somatic tissues (digestive tract, heart, liver, gills, kidney, and retinal cells, among others)		+/+	+/+	+/+	+/+

Note: Tissue presence of mRNA and protein registered using (−), (+), and (++) symbols according to the different levels observed: (−) absent (+), slightly present and (++) highly present.

polygonal shape appearing rounded and basophilic (sighs of cellular atrophy). Most of the hepatocytes also appeared with pyknotic nuclei (Figure 3(C)). Dilatation of the vasculature, congestion of blood vessel, as well as focal or extensive necrosis and apoptosis patterns, located mainly in hepatocytes, were also observed in fish exposed to malathion.

The intestine of the exposed fish presented histopathological lesions that affected the absorptive epithelium (enterocytes), consisting of epithelial desquamation, cell vacuolization and nuclear depolarization with pyknotic nuclei. The absorptive cells also displayed increased lipid deposits (steatosis) within its cytoplasm (Figure 3(D)–(H)).

The most noticeable histopathological alteration related to malathion exposure in the gills was a structural disorganization of the central core of the cartilage. The central zellknorpel that supports the branchial filaments showed considerable disorganization with loss of its structural integrity. While in the controls, the secondary lamellae were oriented parallel to each other and perpendicular to the axis of the filament, in the gills of fish exposed to malathion, the characteristic branchial anatomical structure was not maintained, due to the disorganization of the central axis of cartilage support, and gill filaments and lamellae appeared conglutinated (Figure 3(I),(J)). In parallel, sighs of hyperplasia, hypertrophy and lamellar fusion were detected, due to the exposure to malathion. In these fish, the secondary lamellae lost their typical network of pillar cells- capillaries and sighs of aneurisms and congestion of the blood vessels were also noted (Table 3).

In the kidney, exposure to malathion caused tubular dilation with atrophy of the epithelial cells of the renal tubules, dilation of the glomerular capillaries, and shrinkage of the glomerulus with enlargement of the Bowman's space (Figure 3(K)–(M)). Furthermore, pyknotic nuclei in internal hematopoietic tissue were present in fish exposed to malathion (Table 3).

The histopathological changes detected in the heart after exposure to malathion consisted of a strong disorganization of the cardiac musculature, showing signs of congestion and atrophy. The cardiac fibers showed an evident vacuolar degeneration and began to separate from each other, with the consequent increase and dilation of the myocardial spaces. In some exposed fish, hemorrhagic areas and signs of hemolysis were observed, as well as the presence of aggregates of inflammatory cells located in focal areas of the heart (Figure 3(N)–(P), Table 2).

PCNA antibody displayed positive affinity in most organs-systems and cell tissues of control Senegalese sole. In exposed specimens, an increase in PCNA-positivity have been evidenced in the liver, gills, and in the digestive tract of *S. senegalensis* exposed to the highest malathion concentration (Figure 4(A)–(F), Table 4). On the other hand, the detection of apoptotic cells using TUNEL technique revealed an increase of cell apoptosis in the intestinal epithelium, gills, and skin, as well as in the liver and chondrocytes of the skeletal system, in exposed specimens compared to the controls (Figure 4(G)–(P), Table 4).

4 | DISCUSSION

4.1 | Type "B" esterases and CYP1A biomarker responses

In this study, we have found that exposure to malathion caused a significant reduction in AChE and CbE activities, in those concentrations close to the LC_{50} values ($72hLC_{50}$: 22.94 μg/L), which was previously calculated for this flatfish species.[42] After exposure to malathion, the inhibition of the CbE activity was significantly greater than the inhibition of AChE, although it followed a very similar trend. Therefore, exposure of Senegalese sole larvae to 6.25 μg/L of malathion ultimately resulted in an inhibition of approximately 84%, 40% and 66% for the CbE, and an inhibition of 52, 38 and 49% for the AChE, at 13, 20, and 30 dph, respectively. As the activity of the CbE measured

TABLE 3 Prevalence of histopathological lesions in Senegalese sole specimens exposed to malathion

Organ	Control			1.56 µg/L			3.12 µg/L			6.25 µg/L		
Liver	13d	20d	30d	13d	20d	30d	13d	20d	30d	13d	20d	30d
Atrophy	0	0	0	0	11.1 ± 6	22.2 ± 5	22,2 ± 5	33.3 ± 10	33.3 ± 10	66.7 ± 26	77.8 ± 16	88.9 ± 13
Vasculature dilation	11.1 ± 6	0	11.1 ± 6	11.1 ± 6	11.1 ± 6	22.2 ± 5	22.2 ± 5	33.3 ± 10	44.4 ± 4	88.9 ± 13	100	100
Pyknotic nuclei	0	0	0	0	11.1 ± 6	22.2 ± 5	33.3 ± 10	44.4 ± 4	55.6 ± 10	77.8 ± 16	88.9 ± 13	88.9 ± 13
Necrosis	0	0	0	0	11.1 ± 6	11.1 ± 6	22.2 ± 5	33.3 ± 10	44.4 ± 4	66.7 ± 26	77.8 ± 16	88.9 ± 13
Intestine	13d	20d	30d	13d	20d	30d	13d	20d	30d	13d	20d	30d
Epithelial desquamation	0	0	0	0	33.3 ± 10	33.3 ± 10	33.3 ± 10	44.4 ± 4	55.6 ± 10	44.4 ± 4	77.8 ± 16	88.9 ± 13
Cell vacuolisation	0	0	0	0	33.3 ± 10	44.4 ± 4	33.3 ± 10	33.3 ± 10	66.7 ± 26	44.4 ± 4	77.8 ± 16	100
Nuclear depolarization	0	0	0	0	33.3 ± 10	33.3 ± 10	33.3 ± 10	44.4 ± 4	55.6 ± 10	55.6 ± 10	77.8 ± 16	100
Steatosis	0	0	11.1 ± 6	22.2 ± 5	33.3 ± 10	55.6 ± 10	33.3 ± 10	55.6 ± 10	66.7 ± 26	44.4 ± 4	66.7 ± 26	88.9 ± 13
Gills	13d	20d	30d	13d	20d	30d	13d	20d	30d	13d	20d	30d
Cartilage disorganization	0	0	0	0	11.1 ± 6	33.3 ± 10	33.3 ± 10	44.4 ± 4	66.7 ± 26	44.4 ± 4	66.7 ± 26	100
Lamellar fusion	0	0	0	0	22.2 ± 5	33.3 ± 10	33.3 ± 10	33.3 ± 10	66.7 ± 26	55.6 ± 10	66.7 ± 26	100
Congestion of vessels	0	0	0	0	22.2 ± 5	33.3 ± 10	33.3 ± 10	33.3 ± 10	44.4 ± 4	55.6 ± 10	66.7 ± 26	100
Kidney	13d	20d	30d	13d	20d	30d	13d	20d	30d	13d	20d	30d
Tubular degeneration	0	0	0	0	33.3 ± 10	33.3 ± 10	22.2 ± 5	33. ±10	55.6 ± 10	33.3 ± 10	66.7 ± 26	77.8 ± 16
Shrinkage of gromerulus	0	0	0	11.1 ± 6	33.3 ± 10	44.4 ± 4	33.3 ± 10	44.4 ± 4	55.6 ± 10	77.8 ± 16	77.8 ± 16	100
Pyknotic nuclei	0	0	0	0	22.2 ± 5	33.3 ± 10	22.2 ± 5	33.3 ± 10	33.3 ± 10	22.2 ± 5	66.7 ± 26	77.8 ± 16
Heart	13d	20d	30d	13d	20d	30d	13d	20d	30d	13d	20d	30d
Musculature disorganization	0	0	0	11.1 ± 6	11.1 ± 6	33.3 ± 10	33.3 ± 10	44.4 ± 4	66.7 ± 26	55.6 ± 10	66.7 ± 26	100
Atrophy	0	0	0	0	22.2 ± 5	33.3 ± 10	22.2 ± 5	33.3 ± 10	55.6 ± 10	33.3 ± 10	77.8 ± 16	100
Vacuolar degeneration	0	0	0	11.1 ± 6	22.2 ± 5	33.3 ± 10	33.3 ± 10	33.3 ± 10	66.7 ± 26	33.3 ± 10	77.8 ± 16	100
Miocardial spaces dilation	0	0	0	22.2 ± 5	33.3 ± 10	33.3 ± 10	33.3 ± 10	55.6 ± 10	66.7 ± 26	55.6 ± 10	88.9 ± 13	100

Note: Data presented as percentages (means±SD) from three replicate tanks/treatment.

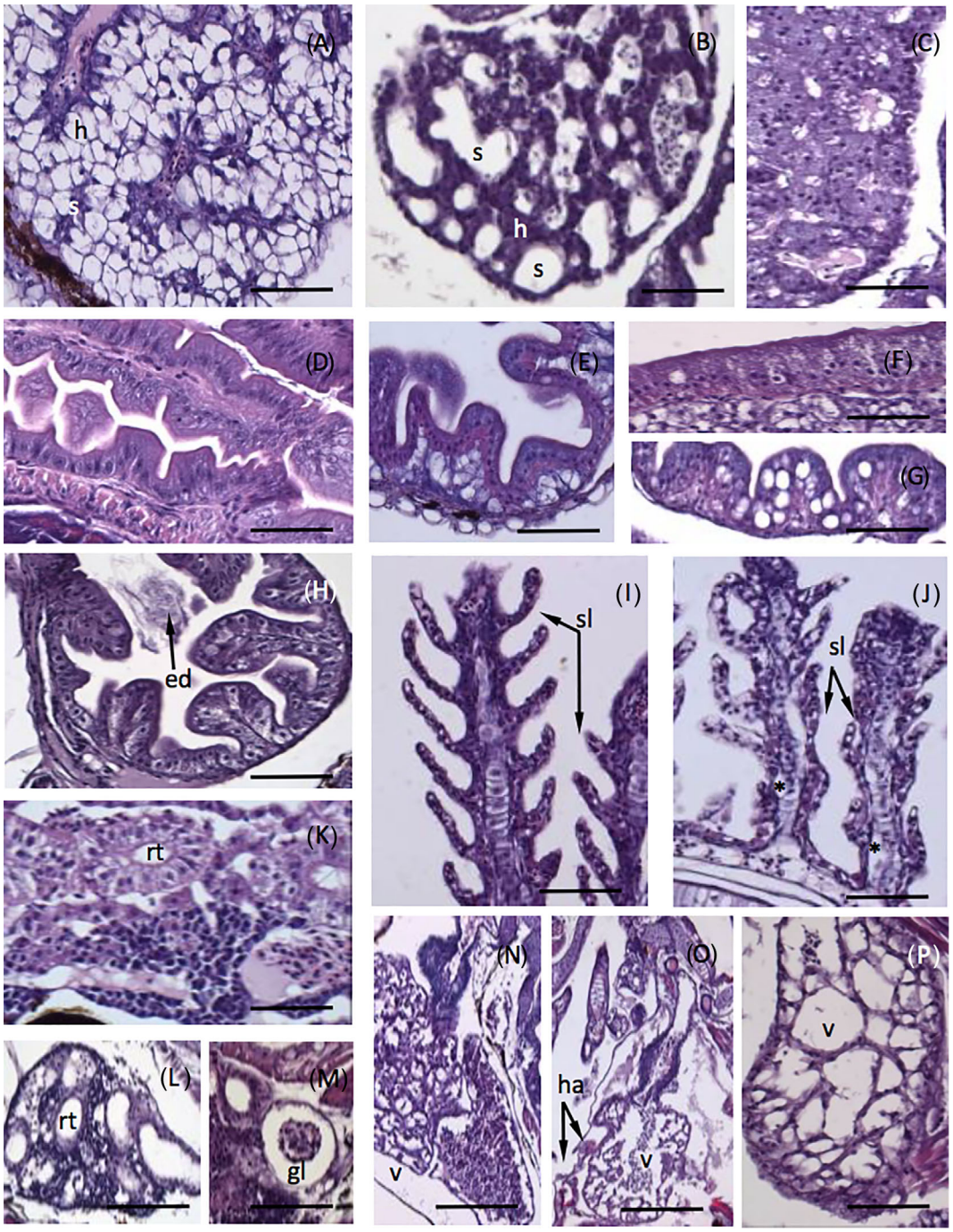

FIGURE 3 Histological sections of different organs and tissues of controls and malathion exposed Senegalese sole at 30 dph (6,25 μg/L). A to C, Liver: (A) hepatic parenchyma from controls showing normal distribution of hepatocytes (h) and sinusoids (s), (B) sinusoids (s) dilation, (C) hepatocyte (h) shrinkage and nuclear pyknosis in malathion exposed specimens. (D)–(H), Intestine: (D) histological sections of intestine from controls showing normal appearance of enterocytes, (E) enterocyte vacuolization, (F) nuclear depolarization with pyknotic nuclei, (G) lipid droplets disposition (steatosis) within enterocyte cytoplasm, and (H) cellular atrophy and epitelial desquamation (ed) in malathion exposed, Senegalese sole, specimens. (I) and (J), gills: (I) normal structure of gill filaments and secondary lamellae (sl) in control Senegalese sole at 30 dph, (J) disorganization of secondary lamellae (sl) and Zell-knorpel necrosis (*) caused by malathion exposure. Note also a moderate dilation of the vascular system within the gill arch and a discrete lamelar fusion in the top of the affected filament. (K)–(M), kidney; (K) normal architecture of renal tubules (rt) from controls, (L) vacuolization of interstitial hematopoietic tissue and severe tubular dilation with atrophy of the endothelia of the majority of the renal tubules (rt), (M) note also a severe glomerular (gl) shrinkage in malathion exposed fishes. (N)–(P), heart: (N) heart chambers from controls, (O) and (P) endocardial dilation and severe degeneration of the myocardial cells from ventricle (v). Note the presence of some haemorrhagic areas (ha) within the ventricle (v) of malathion exposed fish. Scalebars represent: (A)–(C), (H), (L), (M), 25 μm; (D)–(F), (G), (N), (O), 100 μm; (I)–(K), (P), 300 μm

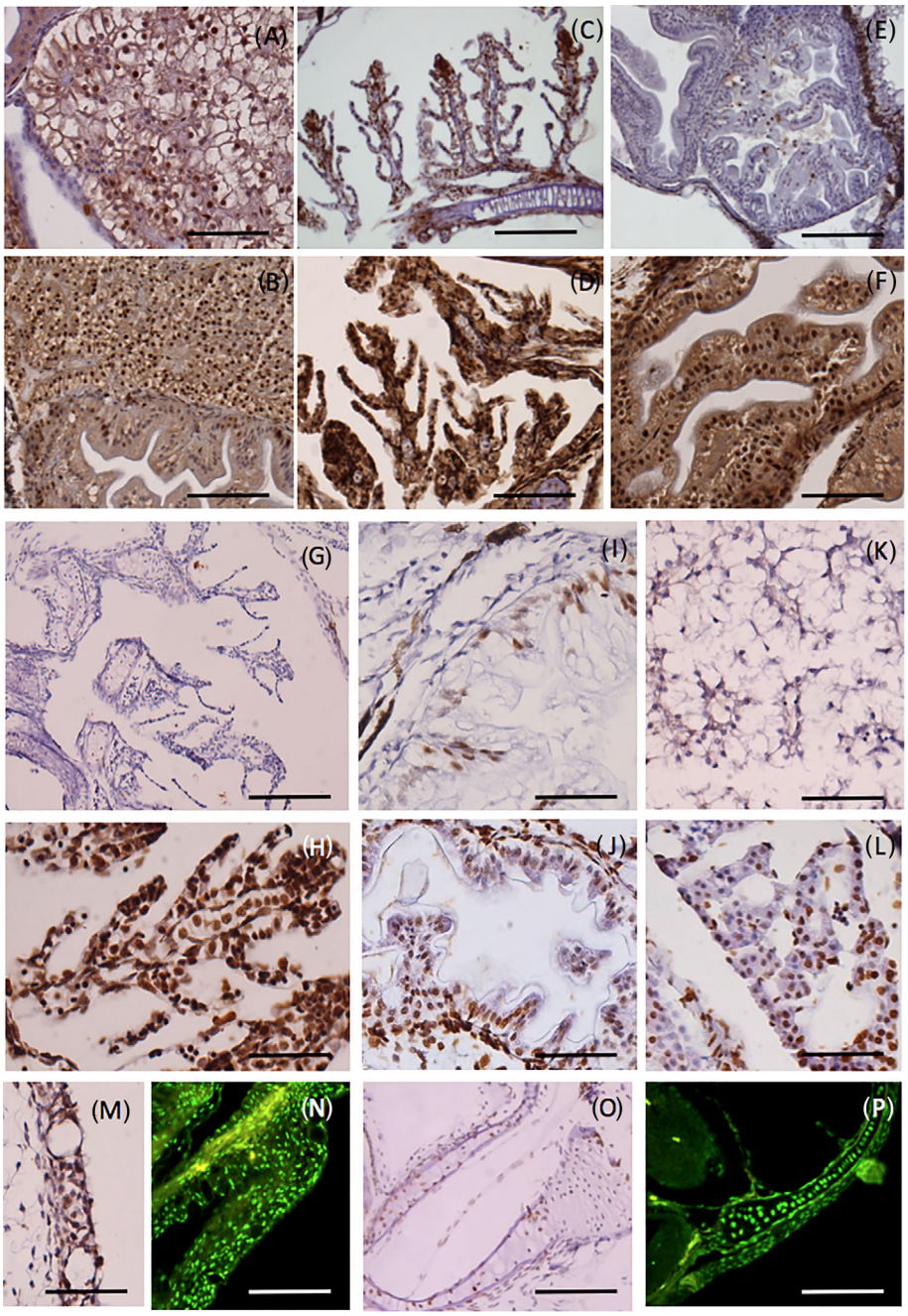

FIGURE 4 Immunohistochemical localization of PCNA in control and malathion exposed (6.25 μg/L, 30dph) Senegalese sole individuals: liver from control (A) and malathion (B) exposed fish, gills from control (C) and malathion (D) treated specimens, gut from control (E) and exposed (F) Senegalese sole at 30 dph. Scalebars represent: (A), (D), (F), 100 μm; (B), (C), (E), 25 μm. Apoptotic cells detection by TUNEL assay in control and 30 dph Senegaleses ole larvae exposed to malathion (6.25 μg/L): gills (G) from control and malathion exposed (H) fish, gut from controls (I) and from malathion (J) exposure, liver from controls (K) and malathion exposed (L) Senegalese sole, skin from controls (M) and exposed (N) fish and chondrocytes from skeletal tissue of controls (O) and malathion exposed (P) fish. Scalebars represent: (G), (I)–(P), 100 μm; H, 300 μm

TUNEL/PCNA		Control	1.56	3.12	6.25
Liver	Hepatocytes	−/+	−/+	+/+	++/++
Gills	Epithelial covering	−/+	−/+	+/+	++/++
	Cartilague chondrocytes	−/+	−/+	+/+	++/+
Digestive tract	Enterocytes	+/+	+/+	+/+	++/++
Skin	Malphigian layer	+/+	+/+	+/+	++/++

TABLE 4 Degree of apoptosis and cell proliferation (TUNEL/PCNA) in control and malathion treated fish at 30 dph

Note: Apoptosis and cell proliferation registered using (−), (+) and (++) symbols according to the different levels observed: (−) absent (+), slightly present and (++) highly present.

by the hydrolysis of PSA was lower than the activity of cholinesterase measured by using ASCh as sustrate[37] and since malathion may not be a more potent inhibitor of CbE than of AChE, the CbE, appears to be able to provide slight protection against AChE inhibition after exposure to malathion in this flatfish.[55] However, no significant changes were detected in the expression levels of the mRNA Ache

transcripts and proteins, such as was detected by q-PCR, ISH, and immunochemical approaches, in Senegalese sole exposed to malathion. Similar results were detected by Schmitt et al.[58] in embryo-larval stages of the zebrafish, *Danio rerio*, under malathion exposure. Although an inverse correlation between gene expression and enzyme activity has been hypothesized,[59] an absence of a relationship between the two biological endpoints has been increasingly reported in the literature.[60–62] In this sense Welki et al[62] pointed out the difficulty of stablishing a direct correlation between gene expression and enzymatic activity due to the complexity of interactions between transcriptomic RNA processes and the activity of enzymes.

In vertebrates, AChE and CbE activities are inhibited by different OP insecticides, such as malathion, chlorpyrifos, and so forth.[13,16,17,63,64] The inhibition of AChE activity is related to the toxic action mechanisms of many insecticides and, therefore, this enzymatic inhibitory effect is often used as a biomarker of OPs exposure and of physiological alterations in organisms exposed to pesticides.[21] CbE have a higher affinity than AChE for some OPs, and it has been suggested that CbE acts as a "sink" for OPs, thus protecting the organism against the toxicity of organophosphorus insecticides.[28] The parallel nature of the dose-dependent increases in the pattern of inhibition of CbE and AChE, and the mortality responses recorded in different organisms, might suggest that inhibition of CbE by OPs pesticides could represent greater availability of OPs to inhibit AChE, resulting in increased sensitivity.[13,25,64]

In Senegalese sole larvae, both "B" type esterases (cholinesterase and carboxylesterase) exhibited a dose–response relationship with similar enzymatic patterns, displaying decreasing activities by increasing malathion concentrations and taking into account higher baseline levels for AChEs> CbE>>> BChE as recorded in controls and in Senegalese sole exposed to the pesticide malathion. In sea bass, *Dicentrarchus labrax*, *S. solea* and *S. senegalensis*, AChE was expressed mainly in the brain and muscles. Other tissues such as the kidney, gonad and liver also expressed AChE activity, although in these organs, this enzyme is likely to have other functions besides neurotransmission. CbE was expressed mainly in the liver, the principal organ of detoxification and processing of xenobiotics, but it can also be detected in kidneys, gonads, and gills.[16,38,39] As evidenced in the present study, in the metamorphic and postmetamorphic phases (13–30 dph), a very low contribution of BChE activity was observed in the muscle of Senegalese sole,[38] as opposed to the seabass.[39] Recent studies carried out in Senegalese sole juveniles revealed that the half maximal inhibitory concentration (IC50) values for muscular AChE and hepatic CbE exposed to chlorpyrifos and its oxon-metabolite were even lower than those of malathion/malaoxon, confirming the oxon form as the most toxic, a high species-sensitivity to these pesticides, and a protective role of hepatic CbE against toxicity of pesticides.[16,25,31,38] In Nile tilapia, the in vivo toxicity of malathion was reflected in the surprising inhibition (90%) of AChE activity in the brain. In contrast, liver activities of CbE, not specific for malathion, were inhibited with less intensity and showed lower reduction rates, between 56% and 36%, respectively.[24] It has been suggested that the toxicity of malathion could be reduced by the action of non-specific serine CbE, which catalyzes the hydrolytic degradation of malathion and/or acts as competitive targets in the inhibition of the malaoxon metabolite, and finally by the action of gluthatione S-transferases, which catalyze the formation of excretable conjugates.[13,16]

It is well known that CbEs, hydrolyze a wide range of exogenous and endogenous esters, and these enzymes provide protection against OPs and other pesticides through two mechanisms: (1) direct hydrolysis of ester bonds in OPs and (2) stoichiometric phosphorylation with the resulting destruction of oxon metabolites, which reduce the amount of OP pesticide available to cause AChE inhibition.[64,65] In *S. senegalensis* larvae, both AChE and CbE activities increased during development, and especially between 20 and 30 dph (metamorphosis and postclimax), when the highest enzyme levels were reached in controls. In fish exposed to malathion (at 6.25 μg/L), significant inhibitions were detected in both AChE and CbE activities, suggesting the rapid oxidation (desulfurization by CYPs) of malathion to the oxon-form. As has been observed in different vertebrates and invertebrates, the CbE has a higher affinity for the bioactivated malathion-metabolite, oxon, and, therefore, this could provide substantial protection against oxons, as these metabolites are generated within organisms.[16,63,64,66]

In *S. senegalensis* during the first month of larval life, the expression levels of Cyp1a transcripts and the immunolocation of constitutive CYP1A proteins were apparently not induced by exposure to malathion. Possibly, the basal levels of constitutive CYP1A, which increased during larval development in most the organic systems and tissues of the Senegalese sole (liver, gills, kidney, digestive, brain, among others), could represent a sufficient amount of CYP proteins, initially, to transform (oxidative desulfurization) malathion into its bioactive form responsible for AChE inhibition, the oxon form, although as indicated in mammals[67] and it has also been suggested in different species of fish[24,25,68–71] other CYP isoforms (i.e., CYP2B, CYP3A, CYP4A), could participate in malathion metabolism in larvae and juveniles of Senegalese sole. In this context, in mammals, different CYP isoenzymes (CYP1A, CYP2B, CYP3A, and others) catalyze oxidative desulfurization reactions and, therefore, may be involved in the activation of malathion.[19] However, it is unlikely that CYP2B and CYP2C isoenzymes are present in Solea species,[26] as suggested in other fish species, or at least these CYPs do not correspond to their counterparts in humans.[24,25,72] In fish species, there is possibly no strict functional homologue of mammalian CYP (i.e., CYP2B) that may be involved in malathion metabolism.[19,20] Furthermore, it has also been suggested that CYP1A is not significantly involved in the activation or detoxification of hepatic malathion.[24,25,73] In fact, for example, in Nile tilapia, the participation of CYP1A in malathion metabolism has been ruled out, since a 40fold induction did not alter the toxicity of malathion, in lethal toxicity tests or by inhibition of AChE activity.[24] In other fish species (e.g., rainbow trout, salmon), malathion, and other pesticides (e.g., sphevalerate) had no effect on Cyp1a mRNA expression levels, protein levels, or EROD catalytic activity.[25,73] In contrast, in cyprinid species, chlorpyrifos slightly increased CYP1A protein levels, after 4 days of exposure by water.[74] However, alterations in the levels of CYP1A proteins induced by OPs are small, suggesting that OPs are weak regulators of CYP1A proteins in fish.[25]

Interestingly, aromaticity and planarity are generally considered two important structural characteristics for the chemical induction of CYP1A.[75] CYP1A inducers with these chemical and structural characteristics, include flat aromatic hydrocarbons, such as dioxins, benzopyrene, β-naphthoflavone, among other lipophilic contaminants.[13,75–77] Since OP-malathion does not contain chlorinated aromatic rings or exhibit planarity, it is not expected to induce CYP1A.[78] Moreover, it has also been pointed out that the main effect of pesticide OPs on the regulation of the CYP1A complex system would be the catalytic suppression of its enzymatic activity/EROD.[24,25,79]

4.2 | Somatic histopathological alterations

In our study, the exposure to malathion affected to a greater or lesser extent, most of the organ systems and tissues of Senegalese sole larvae and juveniles, in a dose-dependent manner. Malathion-induced histopathological alterations, reduced the general healthy state and welfare of exposed fish as well as its development and growth, particularly affecting the critical processes of metamorphosis, eye-migration, pigmentation, cranial remodeling, thyroid axis, among other disorders, as has been recently described on this flatfish.[8,42]

It has been pointed out that malathion effects and histopathological damages, could be mediated by both apoptosis and necrosis pathways.[80,81] In this sense, Chen et al.[82] in carp cell lines treated with malathion detected cytotocicity and increased apoptotic processes. Interestingly, in malathion exposed fish, variable unbalanced patterns of cell proliferation versus cell death or apoptosis rates were detected in several target organ-systems and tissues. DNA damage, evaluated as an increase in positive cells was detected in some tissues like liver, gut, and gills (chondrocytes) by using TUNEL assay. Moreover, with the highest dose of malathion tested (6.25 μg/L), most of the organs and tissues of Senegalese sole showed different cellular damage and histopathological alterations, particularly during the metamorphic phase (13–20 dph) and juveniles.

The most frequent histopathological alterations detected in the gills of the Senegalese sole exposed to malathion showed a general disorganization of the central cartilaginous axis that supported the entire gill structure. As a consequence, the gills lose their typical organization of secondary sheets typically found in the controls: secondary sheets normally arranged parallel to each other and perpendicular to the axis of the filament, and are replaced by an agglutination of their own sheets. In parallel, there was an increase in apoptotic chondrocytes (TUNEL positive cells) within the extracellular matrix that forms the cartilaginous structure of the support axis. Different authors pointed out the harmful effects of malathion on fish support tissues.[83–85] Recently, Ortiz-Delgado et al.[39] in early larval stages of Senegalese sole indicated that malathion induced reduction of collagen fibers of skeletal structures and changes in collagen composition. Ho and Gibson[86] on chicken embryos exposed to malathion reported degenerative necrosis of the cartilage with a reduction of its extracellular matrix and a progressive degeneration of the chondrocytes. Other branchial alterations, such as epithelial lifting, dilation and swelling up of the secondary lamellar epithelium, detachment of pillar cells, hyperplasia, hypertrophy, lamellar fusion and sighs of necrosis and apoptosis, affected the respiratory epithelium that covers both the primary and secondary lamellae of the Senegalese sole exposed to malathion. Morphological alterations of the pillar cells can have several secondary consequences on the osmoregulation capabilities and gaseous exchange mechanisms, causing several physiological disorders.[87,88] The hyperplasic condition characterized by cellular proliferation (increased PCNA immunoreactive cells) was detected in the interlamellar region of the respiratory lamellae of malathion exposed Senegalese sole specimens. According to different authors,[89,90] separation and dilation of lamellar epithelium or hyperplasia of gill epithelia could be a defense response of circulatory system against the pollutants. However, this hyperplasic condition can also alter the respiratory functions. Moreover, cell proliferation of secondary lamellar filaments and lamellar cell hypertrophy decreased the spaces between lamellae and causes its fusion as a protective response, producing decreases in free gas exchange.[89,91,92] According to Dutta et al.,[93] the rupture of the branchial epithelium may be considered a direct dose dependent deleterious effect of pesticides, while hyperplasia, lamellar fusion, and mucous hypersecretion could be signs of the gill defensive responses.

Most xenobiotics, such as OP insecticides and other contaminants, are biotransformed in their metabolites mainly by the liver through various enzymatic systems, and as a consequence of this process, the liver undergoes different levels of tissue and cellular alterations and damages, as it has been described in many other species of fish exposed to different xenobiotics.[13,77,94–96] In the liver of Senegalese sole exposed to malathion, degenerative and dispersed processes were observed, with a prominent enlargement of sinusoids, dilation, hemorrhages and necrosis. These results are similar to previous ones that revealed different histopathological alterations induced by pesticides in the liver of different fish species, including *D. rerio*,[97,98] *H. fossilis*,[99] *Clarias gariepinus*,[100] *Channa punctata*,[101] and *L. rohita*..[102] It has been pointed out that when the liver is damaged, excessive amounts of blood flow to the liver blocking the sinusoids. In this way, blood flows from the hepatic artery and veins into the central vein, and the sinusoids dilate to facilitate this blood flow in damaged hepatic tissues.[103]

The histopathological lesions showed in the intestine of Senegalese sole exposed to malathion were mainly located in the mucosal fold and consisted of epithelial desquamation, nuclear depolarization with pyknotic nuclei and cellular vacuolization. Similar results were observed by Braunbeck and Appelbaum[104] in the intestine of carp exposed to endosulfan which provoked disturbances of intestinal absorption. Cengiz et al.[105] reported oedema, degeneration, accumulation of lymphocytes in the *lamina propria*, pycnotic nuclei, and necrosis in the intestine of *Gambusia affinis* exposed to pesticides. According to Desai et al.,[106] degenerative and necrotic changes observed in the mucosa layer of the Senegalese sole exposed to malathion may be due to a direct toxic effect of the pesticide on the cells, to an accumulation of acetylcholine in the tissues or to a reduction in oxygen supply. Cellular vacuolization detected in the mucosal fold correspond to an excessive lipid deposition (steatosis) within the

enterocyte cytoplasm. The vacuolization might indicate an imbalance between rate of absorption and rate of release of substances within enterocyte cytoplasm.[107] As previously discussed in other contaminated fish,[108] lipidosis or steatosis could be the morphological expression of a blockage in the metabolism of triglycerides due to a defective synthesis of very low density lipoproteins, which are involved in the transport and mobilization of triglycerides from the absorptive enterocytes to other tissues.

PCNA is located in sites of rapid cellular proliferation, and also plays a role in DNA repair and apoptosis by interacting with different partner proteins.[109] In our study, increased PCNA-positivity have been detected in the mucosal intestinal fold of Senegalese sole exposed to malathion, being it related, as in other fish species, with a higher rate of cell renewal of the mucosal fold under contaminant exposure.[110]

On the other hand, lesions in the kidney of fish exposed to contaminants can be expected to be frequent, as it receives a large amount of post-branchial blood. In our study, degeneration of renal and collecting tubules, shrinkage of glomeruli, enlargement of Bowman's space, vacuolization and necrosis of tubular cells were the main histopathological lesions in kidney of Senegalese sole expecimens after malathion exposure. Similar histopathological records were described by Cristina et al[111] in kidney tissue of common carp exposed to 50 μg/L malathion during 72 h exposure. Once the toxicant has reached the renal tissue, induced pathological changes could indicate an alteration of the physiology and functions of this organ that may alter the water balance and the maintenance of a stable internal environment of exposed fish, as was suggested by Deka and Mahanta[80] and Cristina et al.[111]

The investigations related to the effects of pesticides on cardiac muscle are scanty. In current research, alterations in heart muscle had been observed in *S. senegalensis* fish under sublethal malathion treatment. Myocardial muscle fibers showed vacuolar degeneration and atrophy and began to separate from each other, with the consequent increase and dilation of endocardial spaces. Similar effects were detected on heart muscle fibers in *Channa punctatus* fish after exposure to sublethal concentrations of malathion (0.8 mg/L) for 96 h. In parallel to the alteration in the structure of the heart muscle fibers, the researchers also observed a decrease in the biochemical content such as proteins, lipids and glycogen in the heart tissue.[112] In any case, the alterations in the heart as a consequence of exposure to malathion could represent a negative impact on the function of this organ, showing a significant effect on circulation that will alter normal physiology of affected fishes.[113]

As it has been previously suggested, in other fish species, the induction of different metabolic alterations (e.g., liver, heart, etc.) could be attributed to the inhibition of several hormonal and enzymatic pathways, when the fish are exposed to different pollutants, like malathion.[112] The OP malathion also altered the notochordal, thyroidal, skeletal, and pigmentary systems and, therefore, an aquatic subacute exposure to malathion can block the process of larval development, metamorphosis, growth and survival, such as was recently reported in Senegalese sole.[8,42]

5 | CONCLUSIONS

The present study reflects that toxicity assays provide adequate information on the sublethal effects of malathion on *S. senegalensis* larval specimens. Malathion exposure inhibited both esterases type B (AChE, CbEs) in a dose and time dependent manner. However, CYP1A and AChE proteins and mRNA transcripts were not altered. Malathion toxicity also provoked histopathological alterations in different organ systems and tissues (liver, gills, kidneys, heart, and others) of Senegalese sole in a time- a concentration-dependent manner. Furthermore, the malathion concentrations that we found to affect type B esterase activity and to induce histopathological alterations in Senegalese sole larvae were below the expected environmental concentrations reported in many other ecosystems and different fish species.[43–45]

ACKNOWLEDGMENTS

This work was funded by Ministry of Science and Innovation (MICIIN) of the Spanish government (Project AGL2010-15951). The authors are grateful to IFAPA for providing biological material and to Juan A. Canales, M. Carmen Carmona and J. Antonio Miquel for their invaluable technical assistance in this work.

Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Juan Bosco Ortiz-Delgado and C. Sarasquete designed and carried out the experiments. Biological samplings were performed by Juan Bosco Ortiz-Delgado and V. Funes. The gene expression analysis was performed by V. Funes and enzymatic activity by Gemma Albendín and E. Scala. Juan Bosco Ortiz-Delgado and E. Scala carried out the histological analyses. Juan Bosco Ortiz-Delgado wrote the original draft. All the authors provided critical feedback and approved the final manuscript. The study was supervised by C. Sarasquete and Juan Bosco Ortiz-Delgado.

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How to cite this article: Ortiz-Delgado JB, Funes V, Albendín G, Scala E, Sarasquete C. Toxicity of malathion during Senegalese sole, *Solea senegalensis* larval development and metamorphosis: Histopathological disorders and effects on type B esterases and CYP1A enzymatic systems. *Environmental Toxicology*. 2021;36(9):1894–1910. https://doi.org/10.1002/tox.23310